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CASE REPORT

An Atypical *Clostridium* Strain Related to the *Clostridium botulinum* Group III Strain Isolated from a Human Blood Culture

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A nontoxigenic strain isolated from a fatal human case of bacterial sepsis was identified as a *Clostridium* strain from *Clostridium botulinum* group III, based on the phenotypic characters and 16S rRNA gene sequence, and was found to be related to the mosaic *C. botulinum* D/C strain according to a multilocus sequence analysis of 5 housekeeping genes.

CASE REPORT

An 83-year-old woman was hospitalized in a hospital geriatric ward in Paris for a cardiac decompensation. She had a mental disorder and a transient motor weakness on the day planned for her relapse, and she received a benzodiazepine treatment. The same day evening, she fell off her bed and had a fracture of the neck of the femur. Two days later, she developed sepsis (fever, hypotension of 11/5, 33,000 leukocytes/mm^3, and C reactive protein level of 30 mg/liter) and received an antibiotic treatment (cefotaxime, 2 g/day, metronidazole, 0.5 g/8 h intravenously [i.v.]). No characteristic symptom of flaccid paralysis was evidenced. Surgery was delayed, and death occurred the following day. A blood culture performed during the septic phase yielded an anaerobic bacterium called strain AIP981.10.

Bacteria were grown in Trypticase yeast extract-glucose-hemin (TYGH) broth in an anaerobic atmosphere at 37°C. Phenotypic identification was performed with reference methods (1), and metabolic end products (volatile and nonvolatile fatty acids) were assayed by quantitative gas chromatography, as described previously (2). Toxicity was tested using a mouse bioassay (3), and cytotoxicity on Vero cells was performed as previously described (4). The 16S rRNA gene sequence was determined as described previously (5) and was compared to all eubacterial 16S rRNA gene sequences available in the GenBank database by using the multisequence Advanced BLAST comparison software from the National Center for Biotechnology Information (6).

Multilocus sequence typing (MLST) analysis was based on five housekeeping genes (the CTP synthetase [CTPs] gene, *glpK*, *rpoB*, *gyrA*, and *dnaK*) (see Table S1 in the supplemental material). Genome sequences of *Clostridium botulinum* D strain 1873 (NZ_ACSJ01000007) were used as the templates for sequence alignment of the clostridial genes, which have been analyzed, and primers were designed for the conserved sequences with Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). *C. botulinum* type A ATCC 3502 and *Clostridium perfringens* strain 13 were used as outgroups in gene analysis (see Table S2 in the supplemental material). In addition, the botulinolysin, *C. botulinum* C2 toxin, and *Clostridium novyi* hemolysin (termed novyilysin) genes, as well as flagellin genes from *C. novyi* A and B, *Clostridium haemolyticum*, and *Clostridium chauvoei* according to reference 7, were investigated (see Table S1). Gene fragments were PCR amplified and sequenced. Sequence alignments and phylogenetic analysis were conducted using Molecular Evolutionary Genetics Analysis (MEGA) software (version 5) (http://www.megasoftware.net) (8). The phylogenetic inference was based on the neighbor-joining distance method (9). Gene trees were constructed by the neighbor-joining method, using the Kimura two-parameter model (10) and bootstrapping algorithms contained in MEGA software (11).

Strain AIP981.10 was a strictly anaerobic, Gram-positive, spore-forming bacillus that produced lipase and protease but not lecithinase. Gas was produced. Tests for catalase, urease, indole from tryptophan, and reduction of nitrates and nitrites were found negative. Hemolysis on sheep blood agar was observed. A commercial gallery (Rapid ID 32A; bioMérieux, Marcy l'Etoile, France) was inoculated and gave the resulting code 4006400000, which does not correspond to a known species. However, these results indicated that AIP981.10 might belong to the *C. botulinum-C. novyi-Clostridium sporogenes* group of bacteria. Major volatile and nonvolatile fatty acids were propionic (35.7 mM), lactic (12.9 mM), and butyric (6.0 mM) acids, with small amounts of 2-hydroxybutyric and 2-hydroxyvaleric acids. Production of propionic acid as a major metabolism end product is characteristic of *C. botulinum* group III, including *C. botulinum* C and D as well as related species, such as *C. novyi* and *C. haemolyticum* (1, 12). Thus, AIP981.10 was tentatively assigned to a *C. botulinum* from group III or a related species.

Strain AIP981.10 was not toxic, as monitored by injection of 1 ml of culture supernatant intraperitoneally into mice, and there was no cytotoxicity on Vero cells. Botulinum neurotoxin (BoNT) A-to-G genes, as well as flagellin genes of *C. novyi* A and B, *C. haemolyticum*, and *C. chauvoei* were not detected by PCR. Among the toxin genes tested, AIP981.10 gave a PCR amplification only with botulinolysin primers (Table 1). Botulinolysin and novyilysin, produced by *C. botulinum* and *Clostridium novyi*, respectively,

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TABLE 1 PCR detection of novyilysin, botulinolysin, C2 toxin, and clostridiolysin S genes in strain AIP981.10, *C. botulinum* C and D, *C. novyi* A and B, and *C. haemolyticum*

Strain	Result for gene coding for:						
	Novyilysin	Botulinolysin	Phospholipase C	C2 toxin component I	C2 toxin component II	C3 exoenzyme	Clostridiolysin S
AIP981.10	−	+	+	−	−	+	−
C. botulinum							
C strain 468	−	+	+	+	+	+	−
D strain 1873	−	+	+	+	+	+	−
C/D mosaic	−	+	+	+	+	+	−
D/C mosaic	−	+	NA[a]	−	−	NA	NA
C. haemolyticum AIP10052^{T}	−	+	+	−	−	NA	NA
C. novyi							
A strain AIP10062^{T}	+	+	+	−	−	−	−
B strain AIP212.86	−	+	NA	−	−	NA	NA

[a] NA, not available.

are related hemolysins from the cholesterol-dependent cytolysin family, which also encompasses *Clostridium tetani* tetanolysin (13). Botulinolysin primers (see Table S1 in the supplemental material) yielded a PCR detection with all of the *C. botulinum*, *C. novyi*, and *C. haemolyticum* strains tested, whereas novyilysin primers were specific to *C. novyi* (Table 1) suggesting that AIP981.10 is more related to *C. botulinum* C and D, *C. novyi* B, or *C. haemolyticum* than to *C. novyi* A. However, AIP981.10 did not contain C2 toxin genes. The 16S rRNA gene sequence from strain AIP981.10 (1,332 bp) clustered within *Clostridium* cluster I, as defined by Collins et al. (14), in the branch containing *C. botulinum* C, *C. botulinum* D, *C. botulinum* C/D and D/C mosaic strains, *C. novyi* and *C. haemolyticum* (Fig. 1) (7, 14, 15). Sequence from AIP981.10 was more related to those of *C. botulinum* C/D and D/C mosaic isolates (99.9% identity) compared to the other *Clostridium* sequences: *C. botulinum* C strain 468 (99.3%), *C. botulinum* D strain 1873 (99.6%), *C. haemolyticum* ATCC 9650 (99.5%), *C. novyi* A (98.8), and *C. botulinum* C strain Eklund (98.1%).

Analysis of the five housekeeping genes and botulinolysin gene gave the same patterns for AIP981.10 as the 16S rRNA sequence phylogeny tree (Fig. 2). AIP981.10 is located on a distinct branch containing *C. botulinum* D/C mosaic strains between two closely related branches—one containing *C. botulinum* C/D strains and another one encompassing *C. novyi* B, *C. botulinum* C strain 468, *C. botulinum* D, and *C. haemolyticum*. Interestingly, the housekeeping gene sequences of *C. botulinum* C strain Eklund clustered within the branch containing *C. novyi* A, which is distantly related to the one containing the *C. botulinum* C strain, 468. These results are in agreement with those from the phylogenomic analysis of Skarin et al. (16), which show that *C. botulinum* C strain Eklund is closely related to *C. novyi* A. The two *C. botulinum* C strains share 97.2% 16S rRNA gene sequence identity, suggesting that strain Eklund might be assigned to a different species. Nucleotide differences in housekeeping gene and botulinolysin gene sequences between AIP981.10 and the *Clostridium* reference strains (Table 2) indicate that AIP981.10 is more related to the mosaic *C. botulinum* D/C strains than to the other *C. botulinum* and *C. novyi* strains.

In addition, whole-genome sequencing of AIP981.10 has been performed with Illumina single-read sequencing technology. Illumina library preparation and sequencing followed standard protocols developed by the supplier (TrueSeq DNA sample preparation). Briefly, genomic DNA was sheared by nebulization, and sheared fragments were end repaired and phosphorylated. Blunt

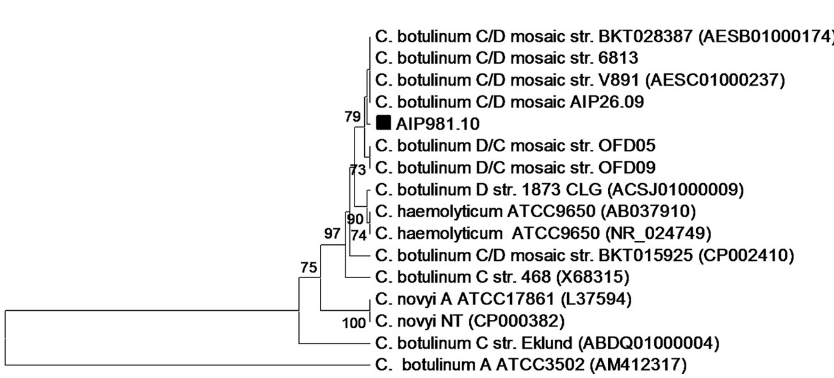

FIG 1 16S rRNA gene analysis. The dendrogram was reconstructed from the nucleotide sequence of the gene by the unweighted-pair group method using average linkages (UPGMA) method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates the genetic distance. The number shown next to each node indicates the percent bootstrap value of 1,000 replicates. Evolutionary analyses were conducted in MEGA5 (8). Strain AIP981.10 is highlighted on the tree by a black square.

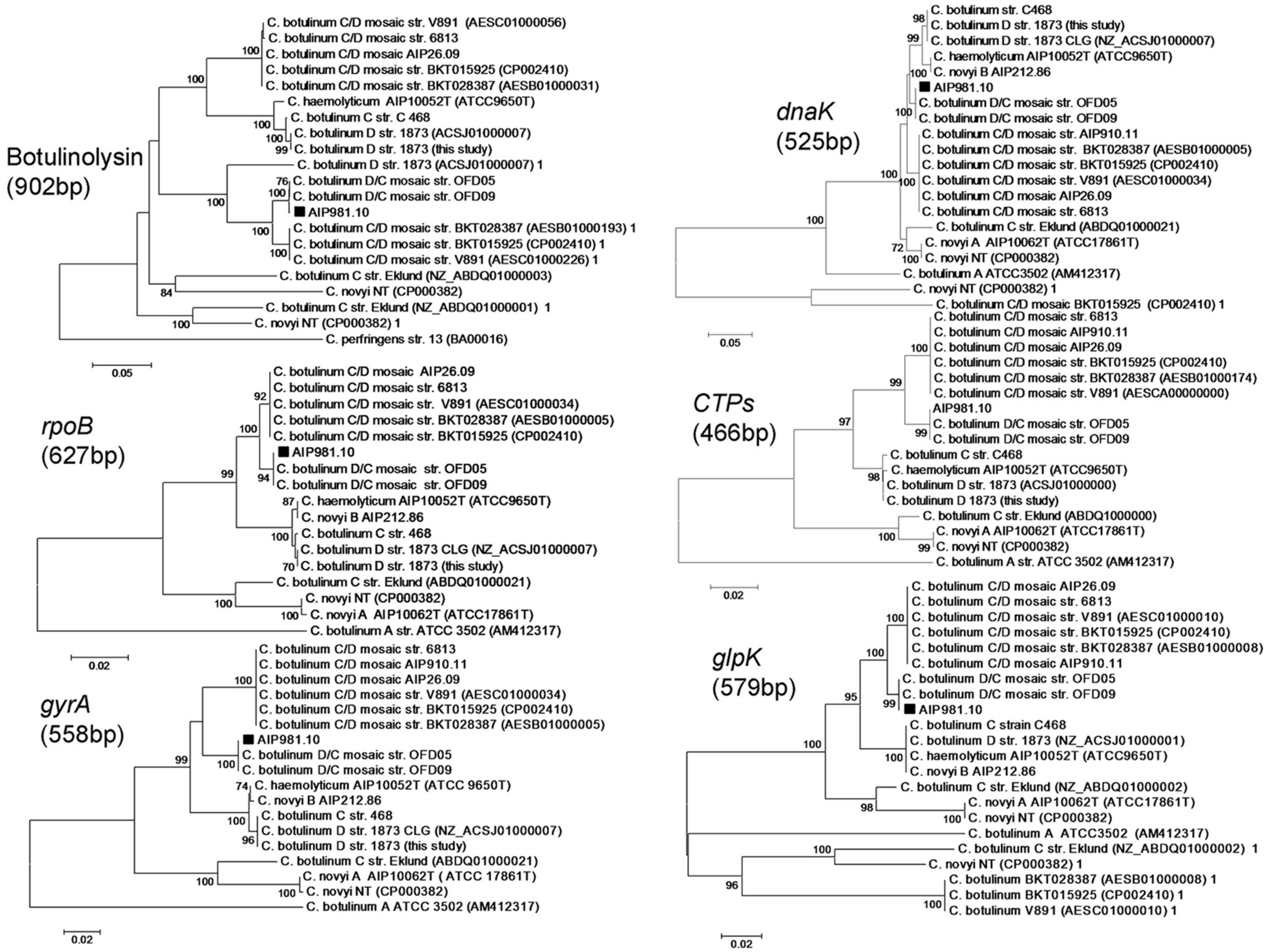

FIG 2 Housekeeping gene and botulinolysin gene analyses. The dendrogram was reconstructed from the nucleotide sequence of the gene by the neighbor-joining method. The genetic distances were computed by using the Kimura two-parameter model. The scale bar indicates the genetic distance. The number shown next to each node indicates the percent bootstrap value of 1,000 replicates. Evolutionary analyses were conducted in MEGA5 (8). Strain AIP981.10 is highlighted on the trees by black squares.

end fragments were A-tailed, and sequencing adapters were ligated to the fragments. Fragments with an insert size of around 400 bp were gel extracted and enriched with 10 cycles of PCR. Hybridization of the library to the flow cell and bridge amplification was performed to generate clusters, and single reads of 50 cycles were collected on a HiSeq 2000 (Illumina, San Diego, CA). After sequencing was complete, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline version 1.7. High-quality filtered reads were assembled using CLC Assembly Cell (CLC bio). A total of 266 contigs ranging from 310 to 109,956 bp were obtained. DNA sequences derived from standard PCR for 16S rRNA genes, botulinolysin genes, and the five housekeeping genes were identical to those from the whole-genome sequencing. Again, the putative botulinum toxin gene and C2 toxin genes have not been identified by sequence similarity searching. However, C3 exoenzyme and phospholipase C genes related to those found in *C. botulinum* C and D (17, 18) have been evidenced in AIP981.10 (Table 1 and 2). An additional toxin gene, coding for clostridiolysin S, has been recently characterized in *C. botulinum*. Clostridiolysin S is related to streptolysin S from *Streptococcus* and is a posttranslationally modified toxin resulting from a cluster of nine genes (*closA*, *closB*, *closC*, *closD*, *closE*, *closF*, *closG*, *closH*, and *closI*) (19). Among the *C. botulinum*/*C. sporogenes* whole-genome sequences available, clostridiolysin S genes have been found only in *C. botulinum* strains from group I and *C. sporogenes*, whereas no related genes have been evidenced in *C. botulinum* group III strains or AIP981.10.

Anaerobic bacteria are responsible for various types of infections, including bacteremia. Bloodstream infections with anaerobes (1 to 17% of positive blood cultures) are mainly due to Gram-negative bacilli. *Clostridium* account for 8 to 18% (up to 46% in some studies) of anaerobic bacteremia in adults (reviewed in reference 20). The most frequently identified *Clostridium* species are *Clostridium perfringens* and *Clostridium septicum*, which are often associated with a dramatic outcome (20, 21). Other toxigenic clostridia might also be involved, such as *Clostridium sordellii* (22), to a lower extent *Clostridium difficile* (23), and one case

TABLE 2 Nucleotide differences between AIP981.10 gene sequences and corresponding sequences from *C. botulinum* C or D and C/D mosaic, *C. haemolyticum*, and *C. novyi* NT

		No. of nucleotide differences between AIP981.10 and corresponding *Clostridium* sequences				
AIP981.10 gene/product (source)	Size (bp)	*C. botulinum* D/C Japanese bovine strain group	*C. botulinum* C/D mosaic avian strains	*C. botulinum* C-*C. botulinum* D-*C. novyi* B-*C. haemolyticum*	*C. botulinum* C strain Eklund	*C. novyi* NT
CTPs (PCR amplicon)	466	0	10	19	49	50
glpK (PCR amplicon)	579	0	9/121[a]	23	35/121[a]	56
rpoB (PCR amplicon)	627	0	6	23	49	56
gyrA (PCR amplicon)	558	0	26	29	72	82
dnaK (PCR amplicon)	525	0	11/217[a]	15	33	24/213[a]
Botulinolysin (PCR amplicon)	902	2	26/154[a]	176	168/195[a]	210
Phospholipase C (gene sequence from WGS)	1,200[b]	NA	55/234[a]	74/77/NA/105[c]	388	394
C3 exoenzyme (gene sequence from WGS)	735[b]	NA	16	16/1/NA/NA[d]	11	NA

[a] Some genomes show two gene copies of *dnaK*, *glpK*, and the botulinolysin and phospholipase C genes that are distantly related. (Differences in both gene copies are separated by a slash.) The corresponding GenBank accession numbers of these genomes are listed in Table S2 in the supplemental material.
[b] Data obtained from the whole-genome sequencing (WGS) of strain AIP981.10.
[c] Nucleotide difference between the phospholipase C gene from AIP981.10 and those from *C. botulinum* C (AB699605), *C. botulinum* D (AB699604), *C. novyi* B (not available [NA]), and *C. haemolyticum* (AF525415), respectively.
[d] Nucleotide difference between the C3 exoenzyme gene from AIP981.10 and those from *C. botulinum* C (X59039), *C. botulinum* D (D17555), *C. novyi* B (NA), and *C. haemolyticum* (NA), respectively.

of *Clostridium tetani* (24). However, nontoxigenic clostridia from the environment, like *Clostridium tertium*, *Clostridium aldenense*, or *Clostridium cadaveris*, are occasionally isolated in blood infections (25–27).

Here we report an atypical *Clostridium* strain related to *Clostridium botulinum* group III, which has been identified in a blood culture. Phenotypic and genotypic analyses indicate that strain AIP981.10 belongs to the physiological group III of *C. botulinum* (reviewed in references 28 and 29), which encompasses *C. botulinum* type C, *C. botulinum* type D, and *C. botulinum* mosaic strains C/D and D/C and which is closely related to *C. novyi* and *C. haemolyticum*. Indeed, the chromosome of the group III *C. botulinum* isolates is highly conserved and is related to that of *C. novyi* (16). However, based on the 16S rRNA sequence, AIP981.10 is on a phylogenetic subbranch distinct from those of the *C. botulinum* and *C. novyi* strains, and multisequence analysis of housekeeping genes shows a close relatedness to the *C. botulinum* D/C mosaic strain and to a lower extent to *C. botulinum* C/D mosaic strain. AIP981.10, which was isolated from a blood culture from a fatal case of infection, was found to not produce lethal toxin. This strain contains botulinolysin, phospholipase C, and C3 exoenzyme genes but not clostridiolysin S genes, supporting that AIP981.10 is phylogenetically related to *C. botulinum* group III strains. In addition, the sequences of the botulinolysin and phospholipase C genes are highly related to those of *C. botulinum* C and D and less similar to those of *C. novyi* and *C. haemolyticum* (Table 2). Both BoNT genes of types C and D and the *C. novyi* alpha-toxin gene (*tcnA*) are localized on phage DNAs that are not integrated into the chromosome. These phages can be easily lost upon subcultures and can be interchanged between *C. botulinum* C or D and *C. novyi* (30–34). Similarly, C2 toxin genes are localized on large plasmids in *C. botulinum* C and D that can be lost or acquired (35). It could be hypothesized that AIP981.10 has lost a phage harboring a BoNT gene during the isolation and subcultures of the strain, and is therefore no longer toxigenic. Indeed a *C. botulinum* type B-like nontoxigenic strain has been isolated from an infant botulism case (36). However, *C. botulinum* C or D strains have been involved only in a few cases of human botulism (37), and no characteristic symptoms of flaccid paralysis were observed in this patient. Alternatively, AIP981.10 could have lost either a phage harboring *tcnA*, a plasmid or another mobile genetic element carrying C2 toxin genes or other unknown toxin gene(s), since the group III *C. botulinum* strains possess various plasmids and mobile elements (35, 38). An environmental nontoxigenic *Clostridium* strain might also be the causative agent in compromised patients, as already evidenced by other nontoxic *Clostridium* species (25–27).

Nucleotide sequence accession numbers. 16S rRNA, phospholipase C, and C3 exoenzyme gene sequences from strain AIP981.10 have been deposited in the GenBank database under accession no. KC589740, KF662728, and KF662729, respectively.

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